Advances in Brief

**ras** Activation of Human Prostate Epithelial Cells Induces Overexpression of Parathyroid Hormone-related Peptide

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Abstract

Immortalized adult and fetal prostate cell lines grown in serum-free conditions produce low levels of parathyroid hormone-related peptide (PTHrP) in the presence of growth factors as assessed by mRNA analysis, PTHrP immunoreactivity, and immunohistochemistry. Subsequent infection of these cells with Kirsten murine sarcoma virus containing an activated Ki-ras oncogene induces at least a 10-20-fold increase in PTHrP expression and production of both adult and fetal immortalized cell lines in the presence of the same growth factors. These results provide the first evidence of direct activation of PTHrP by the ras oncogene in human prostate cells and suggest its potential usefulness as a tumor marker in prostate malignancies.

Introduction

PTHrP is the predominant pathogenetic factor underlying accelerated bone resorption and hypercalcemia in solid tumors (1). PTHrP was shown to be expressed in the majority of prostatic cancers analyzed by immunocytochemistry (2). Although osteoblastic metastases are characteristic of prostatic carcinoma, osteoclastic bone resorption is also accelerated in this condition and may contribute to the morbidity of the disease (3). Furthermore, histomorphometric analyses demonstrate that resorption rate is increased in trabecular bone at sites distant from tumor metastases (3). Accelerated bone resorption seen in metastatic prostatic cancer may, therefore, be secondary to systematically and/or locally produced PTHrP acting alone or perhaps in concert with locally produced cytokines. The mechanisms underlying PTHrP overexpression in human prostate cancer is not known presently but could be related to oncogenic activation because its expression correlates well with increasing tumor grade (2).

The presence of ras mutations has been demonstrated in prostatic carcinomas in Japanese men using PCR and DNA hybridization. Their frequency increased with advanced stages of the tumor and high Gleason score (4). Sixteen of 68 (24%) carcinomas showed ras mutations (4). Mutations in codon 12 of Ki-ras in latent prostatic carcinomas (6 of 22) in Japanese men have been observed, where 13 of 22 tumors also showed focal staining for ras p21 by immunohistochemical staining (5). However, studies in the United State show only a low frequency of ras mutations, which are primarily associated with advanced metastatic disease (6, 7).

Among Japanese men with prostate cancer, ras mutations and the presence of high-risk HPV DNA sequences have been linked at a relatively high frequency (4). In the same group of patients, co-occurrence of ras mutations and HPV infection was more frequent in stage C and D than in stage A and B carcinomas (4, 8). To determine whether Ki-ras could induce PTHrP expression in HPECs, we studied its expression before and after infection of adult and neonatal HPECs with Kirsten murine sarcoma virus containing an activated Ki-ras oncogene.

Materials and Methods

**Cells**

**RWPE-1 Cells.** RWPE-1 cells were isolated from the prostate of a Caucasian man and were immortalized by transfection with HPV type 18 (9). These cells have been extensively characterized to establish their prostatic epithelial origin (10). RWPE-1 cells have a human isozyme phenotype, a chromosome range of 44–51 (10), and show a growth response to androgens. These cells can be induced to express PSA and up-regulate androgen receptor by exposure to the synthetic androgen mibolerone (10). Growth of RWPE-1 cells is stimulated by EGF and basic fibroblast growth factor and inhibited by transforming growth factor β (10). These cells do not form tumors in nude mice (10). On the basis of these characteristics, RWPE-1 immortalized cells retain many of the characteristics of normal cells, such as contact inhibition, anchorage dependence, inability to form tumors in nude mice, and expression of epithelial and prostate-specific markers.

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3 The abbreviations used are: PTHrP, parathyroid hormone-related peptide; HPEC, human prostatic epithelial cell; HPV, human papillomavirus; PSA, prostate-specific antigen; EGF, epidermal growth factor; KGM, keratinocyte growth medium; KBM, keratinocyte basal medium; BPE, bovine pituitary extract; IGF, insulin-like growth factor; GTC, guanidinium thiocyanate; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Activation of PTI-IRP expression derived from a RWPE-1 cell line after infection with Kirsten 0(8)01).

Effect of growth factors on PTHRP secretion. Ki-ras-activated human neonatal (FNC267B1) and adult (RWPE-2) prostate cell lines were grown as described in “Materials and Methods.” Conditioned medium was analyzed in cells before and after Ki-ras activation. PTHRP levels are expressed as picogram equivalents of PTHRP(1–86) per million cells [PTHRP(1–86)/10^6 cells]. Each column represents the mean of triplicate determinations; bars, SE. *, a significant difference from cells prior to Ki-ras activation (P < 0.0001). The data shown are representative of three separate experiments.

**Fig. 2** Effect of growth factors on PTHRP secretion. Ki-ras-activated human neonatal (FNC267B1-ras) and adult (RWPE-2) prostate cell lines were grown as described in “Materials and Methods.” At time 0, the medium was removed and replaced with KBM alone (basal) or a combination of KBM plus EGF (10 ng/ml), 0.4% BPE, or IGF-I (20 ng/ml). Medium was collected at 24 h for measurement of PTHPRP by immunoradiometric assay. Cells were trypsinized and counted. PTHPRP levels were expressed as picogram equivalents of PTHRP(1–86) per million cells [PTHRP(1–86)/10^6 cells]. Each column represents the mean of triplicate determinations; bars, SE. *, significant difference from basal at P < 0.001. The data shown are representative of three separate experiments.

**RWPE-2 Cells.** RWPE-2 cells are tumorigenic and were derived from a RWPE-1 cell line after infection with Kirsten murine sarcoma virus, which contains the activated Ki-ras oncogene (9). These cells have also been extensively characterized. RWPE-2 cells grow in agar and form undifferentiated tumors in nude mice (8, 9). Cells show a growth response to androgen and express PSA in response to treatment with mibololone (8) and ras p21 protein at a high level, as shown by radioimmunoprecipitation assay (9).

**FNC267B1 Cells.** The 267B1 cell line was established by transfecting primary human neonatal prostate epithelial cells with a plasmid containing SV40 early-region genes (11). To obtain the FNC267B1-ras transformed cell line, the 267B1 cells were then transformed with Kirsten murine sarcoma virus containing an activated Ki-ras oncogene, as described previously (12). These cells express ras p21 protein at a high level, as shown by radioimmunoprecipitation assay (12). The FNC267B1-ras cells have a human male karyotype, are aneuploid with chromosome number in the near-diploid range, form colonies in soft agar, and are tumorigenic (12). PC-3 and LNCaP prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD).

**Culture Conditions**

PC-3 and LNCaP cells were maintained in Ham’s F-12 and RPMI, respectively (Life Technologies, Inc., Burlington, Canada) containing 10% fetal bovine serum. RWPE-1, RWPE-2, and FNC267B1 cells were maintained in KGM (Clonetics Corp., San Diego, CA). KGM consists of KBM (Clonetics) containing 0.15 mM of Ca^{2+} supplemented with the following growth factors: 10 ng/ml EGF (Sigma Chemical Co.), 5 μg/ml insulin (Clonetics), 0.5 μg/ml hydrocortisone (Clonetics), and 0.4% (w/v) BPE (Clonetics). Cells were grown to 30% confluence in 10-mm diameter wells in KGM. Following a 24-h incubation in KBM, the medium was replaced at time 0 with KBM containing either 10 ng/ml EGF, 0.4% w/v BPE or 20 ng/ml IGF-I and incubated for 24 or 48 h. Cells were trypsinized, an aliquot was counted, and the remaining cells were centrifuged at low speed (600 × g), rinsed in PBS, and lysed with a mixture of 4 M GTC, 25 mM trisodium citrate, 1 mM EDTA, and 0.1 M β-mercaptoethanol (GTC mixture). GTC extracts were stored at −70°C for subsequent RNA analysis by RT-PCR.

**Assay of Immunoreactive PTHRP in Conditioned Medium**

Conditioned medium, 1.5 ml/well of a 6-well cluster plate, was removed at the appropriate times. Duplicate aliquots of 200 μl were stored at −70°C until assayed. Assays were performed using an immunoradiometric assay specific for PTHRP(1–86) (Diagnostic Systems Laboratory, Webster, TX) as described previously (13). Results were expressed as equivalents of human PTHRP(1–86)/10^6 cells. The detection limit of the assay was 2 pg equivalent of human PTHRP(1–86)/ml of conditioned medium.

**RNA Analysis by RT-PCR**

Total cellular RNA was isolated by acid GTC-phenol chloroform extraction. PTHRP and GAPDH were amplified using primers.
Fig. 3  PTHRP gene expression before and after Ki-ras activation. Human neonatal (FNC267B1) and adult (RWPE-1) prostate cell lines were cultured as described in “Materials and Methods.” At time 0, the medium was removed and replaced with KBM alone (basal) or combinations of KBM plus EGF (10 ng/ml), 0.4% BPE, or IGF-I (20 ng/ml). Cells were trypsinized at 120 h of incubation and analyzed by RT-PCR as described in “Materials and Methods.” Upper panel, the level of expression of the control gene GAPDH. Lower panel, the level of PTHRP expression. The first lane contains the size marker. Lanes 1–10, Ki-ras-activated (FNC267B1-ras and RWPE-2) cells in KBM alone (Lanes 1 and 6), KBM + EGF (Lanes 2 and 7), KBM + BPE (Lanes 3 and 8), KBM + IGF-I (Lanes 4 and 9), and KBM + EGF + BPE (Lanes 5 and 10). Lanes 11–20, FNC267B1 and RWPE-1 cells incubated in KBM alone (Lanes 11 and 16), KBM + EGF (Lanes 12 and 17), KBM + BPE (Lanes 13 and 18), KBM + IGF-I (Lanes 14 and 19), and KBM + EGF + BPE (Lanes 15 and 20).

the following primers: PTHRP upstream primer, TCCAGTCTAAACCCAGCAG; PTHRP downstream primer, GGTGTTCTGTGAGCTACG; GAPDH upstream primer, GGGCGTCTTTTAACTCTG; and GAPDH downstream primer, TGGCAAGTTTTTCTAGACCG. Briefly 1.3 μg of total RNA is reverse transcribed using a reverse transcriptase mix consisting of 2 μl of 10 × PCR buffer (Perkin-Elmer, Montreal, Quebec, Canada), 0.25 μl RNase inhibitor, 2 μl of deoxynucleotide triphosphates (10 mM), 0.5 μl reverse transcriptase, 1 μl of random hexamer primers, and 8.25 μl of water. Tubes are then placed in the thermocycler (Perkin-Elmer) and treated at the following temperatures: 10 min at 23°C, 45 min at 42°C, and 5 min at 95°C. A PCR mix consisting of 8 μl of 10× PCR buffer, 1 μl (100 pmol) of each primer, 0.5 μl of Taq polymerase, and 69.5 μl of water is then added to the tubes, and the DNA was amplified for 25–30 cycles. Analysis is then performed on a 2% agarose gel stained with ethidium bromide and quantified by laser densitometry.

Immunocytochemistry

Cellular content of PTHRP was determined by immunocytochemistry. Cells were seeded in four-chamber glass slides (Life Technologies, Inc.) at a density of 5000 cells per chamber in KBM containing 10 ng/ml of EGF until near confluency. Cells were then fixed in 95% ethanol for 5 min, rinsed with distilled water, and stained with a rabbit antibody to PTHRP(1–34) using a modification of the three-layer peroxidase-antiperoxidase technique (14).

Statistical Analysis

Results are expressed as means ± SE of replicate (at least triplicate) determinations, and statistical comparisons are based on one-way ANOVA or by Student’s t test. A probability value of P < 0.01 was considered significant.

Results

HPEC lines RWPE-1 and RWPE-2 have been characterized extensively. Both cell lines respond to androgens by expression of PSA and up-regulation of androgen receptor, as detected by immunostaining (8, 9).

Effect of Ki-ras Activation on PTHRP Secretion

Human prostatic epithelial cells immortalized with either SV40 or HPV18 were analyzed for immunoassayable PTHRP in the culture medium. There was a small but significant secretion of PTHRP in the culture medium (Fig. 1) following stimulation with KGM for 48 h. However, following Ki-ras activation, we observed a 10–20-fold increase of PTHRP levels in both cell
As seen in Fig. 3, EGF, effect of Ki-ras activation in both neonatal and adult prostatic cells HPECs (Fig. 4), on both PTHRP mRNA and secretion.

Effect of EGF, BPE, and IGF-I on PTHRP Secretion in Ki-ras-activated Cells. Cells were grown as described in “Materials and Methods.” In the absence of exogenous growth factors (KBM alone), PTHRP levels were low in both neonatal (FNC267B1-Ki-ras) and adult (RWPE-2) Ki-ras transformed prostatic cells. However, following stimulation with either EGF, BPE, or IGF-I, there was a 10–20-fold increase of PTHRP secretion after 24 h of incubation (Fig. 2). The effect on PTHRP secretion was accompanied by a significant increase in cell proliferation by growth factors. Ki-ras activation of both RWPE-2 and FNC267B1 resulted in a slight acceleration of cell proliferation (=20%) after stimulation with the various growth factors. All results on PTHRP production were corrected for cell number [PTHRP(1–86)/10^6 cells].

Effect of Ki-ras Activation on PTHRP mRNA Levels. Cells were grown as described in “Materials and Methods,” and the effect of Ki-ras activation in both neonatal and adult prostatic cells was examined. In both cell lines, Ki-ras activation resulted in a dramatic increase of PTHRP mRNA levels, as seen in Fig. 3. EGF, BPE, and IGF-I had a similar effect on PTHRP expression in Ki-ras-activated cells from either adult or neonatal origin.

Immunocytochemical Staining of HPECs before and after Ki-ras Activation. Immunocytochemical studies using a PTHRP antibody directed against PTHRP(1–34) showed that Ki-ras activation resulted in an intense increase of both the intensity of staining and the number of stained cells in both adult and neonatal HPECs (Fig. 4), confirming the dramatic changes on both PTHRP mRNA and secretion.

Discussion

PTHRP production has been described previously in primary cultures of HPECs and is induced primarily by EGF in this model (15). Our present data indicate that Ki-ras activation induces dramatic changes in PTHRP expression and production in two independent systems and contrasts sharply with the low level of production of PTHRP in two widely used prostatic cancer cell lines, PC-3 and LNCaP. Increased expression of ras or ras mutations is apparently associated with advanced disease, high-stage and high-grade cancer involving invasive growth and metastasis (6, 7, 16). Mutations in the ras gene were more frequent in stages C and D than in stages A and B (4). A correlation between ras p21 expression in primary tumors and the presence of nodal metastases has also been reported (16). Although up to 26% of latent prostatic carcinomas in Japanese men show ras mutations, the reported frequency of ras mutations is less than 5% in the United States (4, 17–19). In a recent study in Japan, three of nine prostatic carcinomas showed ras mutations (17). Considerable intratumoral heterogeneity with regard to increased ras expression and ras mutations was reported, where different foci within the tumor contained mutations in K-ras in codons 12 or 61, and seven of nine tumors showed focal staining for ras p21 (17). Two other observations may suggest some role of ras in prostatic carcinoma. In the human prostatic carcinoma cell line PC-3, emergence of drug-resistant cells was associated with increased expression of c-jun, c-myC, and H-ras (20). Furthermore, in a mouse prostate reconstitution model, both epithelial and mesenchymal cells of the urogenital sinus were transfected with ras and myc oncogenes. When these cells were grafted under the kidney capsule, poorly differentiated prostatic carcinomas developed in 90% of the cases (21).

Because PTHRP overexpression is frequently observed in human prostate cancer and correlates also with histological grade (2), we decided to examine whether an association between ras and PTHRP expression may also exist. However, further studies aimed at analyzing concomitantly ras and PTHRP expression in prostatic adenocarcinoma will be necessary to confirm this hypothesis. From the clinical standpoint,
PTHRP overexpression in Ki-ras-activated cells may well play a significant role in accelerated osteoclastic bone resorption observed in the vicinity of osteoblastic metastasis in prostate cancer (3). The present model may, therefore, find exciting new applications aimed at understanding the mechanism of bone resorption in prostate cancer. Furthermore, it has been suggested that PTHRPs may play a role in the development of metastasis in advanced solid cancer (22). Furthermore, it has been shown that Ki-ras-transformed RWPE-2 cells secrete higher levels of urokinase, a key protease involved in invasion by prostate cancer cells, than the parent RWPE-1 cells (23). Consequently, the human model described here may find new applications for the study of skeletal complications of human prostate cancer.

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References


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